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Abstract: • There is controversy as to whether specific natural products play a role in directly mediating antagonistic plant-plant interactions - that is, allelopathy. If proved to exist, such phenomena would hold considerable promise for agronomic improvement of staple food crops such as rice (Oryza sativa). • However, while substantiated by the presence of phytotoxic compounds at potentially relevant concentrations, demonstrating a direct role for specific natural products in allelopathy has been difficult because of the chemical complexity of root and plant litter exudates. This complexity can be bypassed via selective genetic manipulation to ablate production of putative allelopathic compounds, but such an approach previously has not been applied. • The rice diterpenoid momilactones provide an example of natural products for which correlative biochemical evidence has been obtained for a role in allelopathy. Here, we apply reverse genetics, using knock-outs of the relevant diterpene synthases (copalyl diphosphate synthase 4 (OsCPS4) and kaurene synthase-like 4 (OsKSL4)), to demonstrate that rice momilactones are involved in allelopathy, including suppressing growth of the widespread rice paddy weed, barnyard grass (Echinochloa crus-galli). • Thus, our results not only provide novel genetic evidence for natural product-mediated allelopathy, but also furnish a molecular target for breeding and metabolic engineering of this important crop plant.

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Genetic evidence for natural product mediated plant–plant allelopathy in rice

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Summary

- A role for specific natural products in directly mediating antagonistic plant–plant interactions –that is, allelopathy –has been controversial. If proven, such phenomena would hold considerable promise for agronomic improvement of staple food crops such as rice (*Oryza sativa*).
- However, while substantiated by the presence of phytotoxic compounds at potentially relevant levels, demonstrating a direct role for specific natural products in allelopathy has been difficult due to the chemical complexity of root and plant litter exudates. This complexity can be bypassed via selective genetic manipulation to ablate production of putative allelopathic compounds, but such an approach previously has not been applied.
- The rice diterpenoid momilactones provide an example of natural products for which correlative biochemical evidence has been obtained for a role in allelopathy. Here, we apply reverse genetics, using knock-outs of the relevant diterpene synthases (OsCPS4 and OsKSL4), to demonstrate that rice momilactones are involved in allelopathy, including suppressing growth of the widespread rice paddy weed, barnyard grass (*Echinochloa crus-galli*).
- Thus, our results not only provide novel genetic evidence for natural product mediated allelopathy, but also furnish a molecular target for breeding and metabolic engineering of this important crop plant.

Keywords

allelopathy; biosynthetic gene cluster; diterpenoids; momilactones; rice (*Oryza sativa*); weed suppression

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Supporting Information

Additional supporting information may be found in the online version of this article.

Introduction

The suggestion that plants make certain natural products to suppress the growth of their neighbors was first made over a century ago, and has been termed allelopathy (Willis, 2007). However, all of the reported evidence for this phenomenon is essentially correlative, relying on the identification of phytotoxic compounds present at potentially relevant levels (Field *et al.*, 2006). Demonstrating a role for specific natural products in allelopathy has been confounded by the chemical complexity of root and plant litter exudates, as well as the interaction of these compounds with the soil matrix and associated microbes (Belz, 2007). Moreover, difficulties have been encountered in reproducibly showing the presence of natural products associated with allelopathy at phytotoxic levels in the soil (Bais *et al.*, 2003; Duke *et al.*, 2009). Thus, the role of natural products in this process remains controversial (Field *et al.*, 2006). The existence of such allelopathy could be most directly tested by selective removal of the compounds of interest via genetic manipulation, but no such experiments have been previously reported. Even in cases where the appropriate selective reduction in potential allelopathic natural product in isogenic plant lines has been reported (Shimura *et al.*, 2007; Cook *et al.*, 2010).

Rice is arguably the single most important source of human nutrition, providing ~20% of our caloric intake worldwide, requiring widespread cultivation on more than 150 million hectares globally (FAO, 2004). To prevent the large losses in yield that would otherwise occur, rice is traditionally grown in paddies, with flooding to inhibit the growth of weed plants, followed by the subsequent setting of rice seedlings. However, this practice is water and labor intensive. More modern cultivation methods rely on the application of herbicides, which imposes accompanying economical, environmental, and human health costs. Accordingly, for rice and other staple crops there is significant interest in investigation of the secretion of natural products that directly suppress the growth of neighboring plants (Belz, 2007). Yet, surprisingly, the evidence for such allelopathic effects has remained somewhat indirect (Field *et al.*, 2006).

For example, the rice momilactones were originally isolated as natural plant growth suppressants (Kato *et al.*, 1973), and have been hypothesized to serve as allelopathic natural products (Kato-Noguchi, 2004). The importance of the momilactones is highlighted by the presence of a dedicated biosynthetic gene cluster in the rice genome (Fig. 1a) (Wilderman *et al.*, 2004; Shimura *et al.*, 2007), which is an emerging theme in plant secondary metabolism that has been associated with important roles in plant defense (Osbourn, 2010). As labdane-related diterpenoids, momilactone biosynthesis is initiated by dual cyclization reactions sequentially catalyzed by class II diterpene cyclases and class I diterpene synthases (Peters, 2010). In particular, initial cyclization of the general diterpenoid precursor (*E,E,E*)-geranylgeranyl diphosphate (GGPP) to *syn*-copalyl diphosphate (*syn*-CPP) is catalyzed by the rice CPP synthase OsCPS4 (Otomo *et al.*, 2004b; Xu *et al.*, 2004). This is followed by further cyclization of *syn*-CPP to *syn*-pimaradiene by the rice kaurene synthase-like OsKSL4 (Otomo *et al.*, 2004a; Wilderman *et al.*, 2004). Notably, the genes encoding OsCPS4 and OsKSL4 are found close together in the rice genome (Wilderman *et al.*, 2004). Furthermore, this region also contains a dehydrogenase that catalyzes the final step in production of momilactone A (OsMAS), and two closely related cytochromes P450 (CYP99A2&3), one or both of which are required for momilactone biosynthesis (Shimura *et al.*, 2007). More specifically, CYP99A3 recently has been shown to act as a *syn*-pimaradiene oxidase that produces *syn*-pimaradien-19-oic acid, presumably as an intermediate en route to the 19,6-γ – lactone ring of the momilactones (Fig. 1b) (Wang *et al.*, 2011).

Like all other natural products associated with plant–plant allelopathy, the evidence for momilactone function in rice allelopathy is essentially correlative. For example, while there

is a relationship between momilactone B levels and allelopathic effect in various rice cultivars (Kato-Noguchi *et al.*, 2010), these lines differ in multiple traits, and rice root exudates contain other phytotoxic natural products that also are found in rice paddy soil and have been suggested to contribute to allelopathy (Belz, 2007). Moreover, the momilactone biosynthetic gene cluster was not linked to allelopathy by QTL mapping (Jensen *et al.*, 2001). In addition, the momilactones have been suggested to function as antibiotic phytoalexins against the important fungal blast pathogen *Magnaporthe oryzae* (Cartwright *et al.*, 1981; Hasegawa *et al.*, 2010), which can infect rice through the roots as well as shoots (Sesma & Osbourn, 2004), providing an alternative or complementary rationale for the presence of these diterpenoids in root exudates.

Materials and Methods

Insertional mutant rice lines were first identified via the Salk Research Institute Rice Functional Genomic Express Database (signal.salk.edu/cgi-bin/RiceGE). For OsCPS4 (TIGR gene accession Os04g0990), a putative exon T-DNA insertion line in an *Oryza sativa* L. ssp. *japonica* cv Zhonghua 11 background was available (RMD_03Z11UZ33), and seeds obtained from the Rice Mutant Database (Zhang *et al.*, 2006). For OsKSL4 (TIGR gene accession Os0410060), a putative 3′UTR T-DNA insertion line in a ssp. *japonica* cv Hwayoung background was available (PFG_1E-04430.R), and seeds obtained from the Plant Functional Genomics Laboratory (Jeon *et al.*, 2000). In both cases, seeds of the parental wild type line also were obtained.

Homozygous insertional mutant lines were identified from the supplied seeds by the expected loss of gene expression and momilactone production. These analyses were carried out with hydroponically grown 2-wk-old seedlings, with the plants used for RT-PCR analysis of gene transcription following methyl jasmonate induction, performed as previously described (Wilderman *et al.*, 2004; Xu *et al.*, 2004), and momilactone production measured using the root exudates. Briefly, individual surface sterilized seeds were germinated and grown on plastic mesh suspended on 5 ml of sterile water in loosely capped 50 ml tubes in a growth chamber with 12 h light : dark (28 : 24°C) cycles, with sterile water added as necessary to maintain a constant level. After 15 d, the water from individual tubes was passed through No. 2 filter paper, and then loaded onto 0.1 g Bond Elut C18 spin-cartridges (Varian), which were then washed with 1 ml 50% methanol/water and eluted with 0.5 ml 100% methanol. This was diluted with 0.5 ml deionized water for LC-MS/MS analysis using an Agilent 1100 HPLC with coupled ion trap mass spectrometer instrument located in the W.M. Keck Metabolomics Research Laboratory at Iowa State University (USA). Analysis of 10 μl samples was carried out in positive ion mode, using a 0.3 ml min^{-1} flow rate with 70% acetonitrile containing 0.1% acetic acid as the running buffer, and a Supelco Ascentis C18 column (10 cm × 2.1 mm, 3 μm). The momilactones were detected using the base peak (*m/z* = 271 or 269 for A and B, respectively) resulting from fragmentation of the relevant molecular ion (*m/z* = 315 or 331, respectively). Verified homozygous mutant lines (i.e., knock-outs), were then propagated through two generations to provide seeds for the experiments described here.

Magnaporthe oryzae (rice isolate Guy 11) infection assays were carried out as previously described (Sesma & Osbourn, 2004). *Fusarium fujikuroi* infection assays also were carried out as previously described (Wiemann *et al.*, 2010). Allelopathy was assessed in the absence of added nutrients to remove any confounding effects of competition. Accordingly, rice was grown by germination of husked, sterilized seeds on water agar petri plates lined with sterile filter paper at 28°C in the dark. Four-day old seedlings of uniform size were then transferred to sterile disk filter paper moistened with 3 ml sterile water in a petri dish (6 seedlings per 100 mm plate), and grown an additional 3 d under continuous light at room temperature,

with the addition of 1 ml more sterile water every 24 hrs. The biomass of the mutant and parental wild type rice plants was measured to verify that there was no significant difference at this stage of growth (see Supporting Information). Ten lettuce (*Lactuca sativa* cv Black-seeded Simpson) or barnyard grass (*Echinochloa crus-galli* cv Kudiraivali) seeds were then added to each plate, and the resulting seedling lengths measured after 6 or 12 d for lettuce and barnyard grass, respectively.

Results

Based on the availability of an OsCPS4 insertion mutant, we undertook a reverse genetic approach to begin investigating physiological function(s) of the momilactones. A homozygous cps4 knock-out line was identified based on the expected loss *OsCPS4* gene expression, as well as momilactone production (Fig. 2a). Critically, these plants seemed to exhibit normal growth through all stages of development (e.g., Fig. 2b).

To determine if the momilactones, or any other OsCPS4 dependent diterpenoids, were involved in defense against the agronomically devastating rice blast disease caused by *M. oryzae*, the cps4 mutant and parental wild-type lines were infected with a rice isolate of this fungal pathogen. Intriguingly, there was no significant difference in susceptibility of cps4 mutant relative to the parental plants, which was true for infection via either the leaf or root (Fig. 3a). In addition, cps4 mutant plants did not seem to be any more susceptible than wild-type to another important rice root fungal pathogen, *Fusarium fujikuroi*, causative agent of the bakanae 'foolish seedling' disease (Supporting Information Fig. S1). Accordingly, although the fungal elicitor inducible transcription of *OsCPS4* (Okada *et al.*, 2007), and antibiotic activity of the resulting labdane-related diterpenoid natural products (Cartwright *et al.*, 1981; Kodama *et al.*, 1992; Hasegawa *et al.*, 2010), suggests that these will play a role in rice plant defense against some plant pathogens, they do not appear to play a significant role against the tested root fungal pathogens at the levels found in the parental ssp. *japonica* cv Zhonghua 11 wild type plants.

By contrast, there was a striking difference between the effect of the cps4 mutant and wild-type rice in allelopathy bioassays, as other plant species germinated in the presence of the cps4 mutant exhibited significant (P–values < 0.05) increases in seedling length relative to those germinated with the parental line. Given the absence of any supplied nutrients in these assays, this further appears to reflect a direct effect on growth of the seedlings (i.e., rather than an indirect effect due to competition for nutrients). Importantly, these effects were not only observed with the sensitive dicot lettuce (Fig. 3c), but also with the monocot and agronomically important rice paddy weed barnyard grass as well (Fig. 3d).

In addition to momilactones, the diterpene cyclase OsCPS4 is required for biosynthesis of oryzalexin S, as well as other diterpenoid metabolites (Fig. 1b) (Peters, 2006; Morrone *et al.*, 2011). To more precisely investigate the role of the momilactones, we turned to the downstream OsKSL4, which is more specifically dedicated to momilactone biosynthesis, and for which an insertion mutant also was available. Again, a homozygous ksl4 knock-out line was identified based on the expected loss of *OsKSL4* gene expression, as well as momilactone production (Fig. S2).

Consistent with a specific role for the momilactones in rice allelopathy, the ksl4 mutant also exhibited a significant loss of allelopathic effect relative to its parental wild-type line against both lettuce and barnyard grass (Fig. 4). Indeed, the observed loss of allelopathy was even more dramatic than that observed with the cps4 mutant, with increases in lettuce and barnyard grass seedling lengths of >25%, and P-values < 0.0001 for this difference. Given the known variation between various rice lines (Belz, 2007), the increased effect observed

with the ksl4 mutant is presumably due to greater allelopathic effect of its parental wild-type line relative to that for the cps4 mutant.

Assembly of biosynthetic gene clusters, such as that from the rice genome associated with momilactone production (Wilderman *et al.*, 2004; Shimura *et al.*, 2007), is relatively uncommon in plants, and has been associated with important roles for the associated natural products/metabolites in defense (Osbourn, 2010). Notably, while OsCPS4 dependent metabolites such as the momilactones and oryzalexin S have been suggested to serve as phytoalexins against the rice blast pathogen *M. oryzae* (Cartwright *et al.*, 1981; Kodama *et al.*, 1992; Hasegawa *et al.*, 2010), these do not seem to be important in rice plant defense, at least against the tested isolate of the root fungal pathogen *M. oryzae*, as well as *F. fujikuroi*. This highlights the allelopathic activity of the momilactones shown here, which then presumably provided at least part of the positive selection pressure leading to assembly of the associated biosynthetic gene cluster.

In addition to positive selection pressure, it has been suggested that assembly of biosynthetic gene clusters in plants also might be due to production of intermediates with detrimental bioactivity, providing negative selection pressure against inheritance of only early biosynthetic genes (Chu *et al.*, 2011). Indeed, while its subsequent growth and development appear to be normal, the ksl4 mutant exhibits a notable ~2-fold reduction in germination rate relative to its parental wild-type line (Fig. 5). This presumably reflects deleterious effect(s) from accumulation of the OsCPS4 *syn*-CPP product, or a derived metabolite, that occurs in the absence of OsKSL4. For example, there are other KSL (i.e., class I diterpene synthases) that also react with this CPP stereoisomer (Fig. 1b) (Nemoto *et al.*, 2004; Kanno *et al.*, 2006; Morrone *et al.*, 2006, 2011; Xu *et al.*, 2007), and their activity might lead to production of a germination inhibitor. Regardless of exact mechanism, this provides clear counter-selection against inheritance of only OsCPS4, which then provided negative selection pressure for assembly of the observed momilactone biosynthetic gene cluster (i.e., to ensure co-inheritance of OsCPS4 with subsequently acting biosynthetic genes). This is consistent with investigations of other plant biosynthetic gene clusters (Field & Osbourn, 2008), and supports an important role for such dual ‘push-pull’ positive and negative selective pressure in driving their assembly.

Discussion

Previous reports of allelopathy have been based on correlation of plant growth suppression activity with detection of the relevant natural products at phytotoxic levels. Given the complexity of plant root exudates, this leaves in question a direct role for specific natural products in allelopathy (Field *et al.*, 2006). Accordingly, the reverse genetics approach taken here provides novel and more direct evidence for such activity. Specifically, the effects of knocking out either of the relevant diterpene synthases, OsCPS4 and OsKSL4, demonstrates a direct role for the momilactones in the allelopathic ability of rice to suppress the growth of neighboring plants. Furthermore, the presence of a momilactone dedicated biosynthetic gene cluster, whose assembly seems to have been driven, at least in part, by the allelopathic activity of these natural products, highlights the physiological and evolutionary importance of such activity in the rice plant lineage.

Given the potential benefits that allelopathy offers for the cultivation of widely grown crop plants such as rice, such activity has been extensively investigated. Over 10,000 rice lines have been screened for allelopathy, with the finding that ~4% effectively suppress the growth of rice paddy weeds such as barnyard grass (Belz, 2007). The evidence presented here provides a relevant molecular target for breeding and/or metabolic engineering efforts in rice. In particular, increased momilactone production levels can serve as a target for

selective molecular breeding and/or genetic/metabolic engineering. Furthermore, the inducible nature of momilactone biosynthesis in rice (Cartwright *et al.*, 1977; Ren & West, 1992; Nojiri *et al.*, 1996; Kato-Noguchi *et al.*, 2007), suggests that such induction also might be advantageous in promoting the endogenous ability of rice to suppress weed growth. Together, these potential applications provide significant agricultural relevance for the results reported here.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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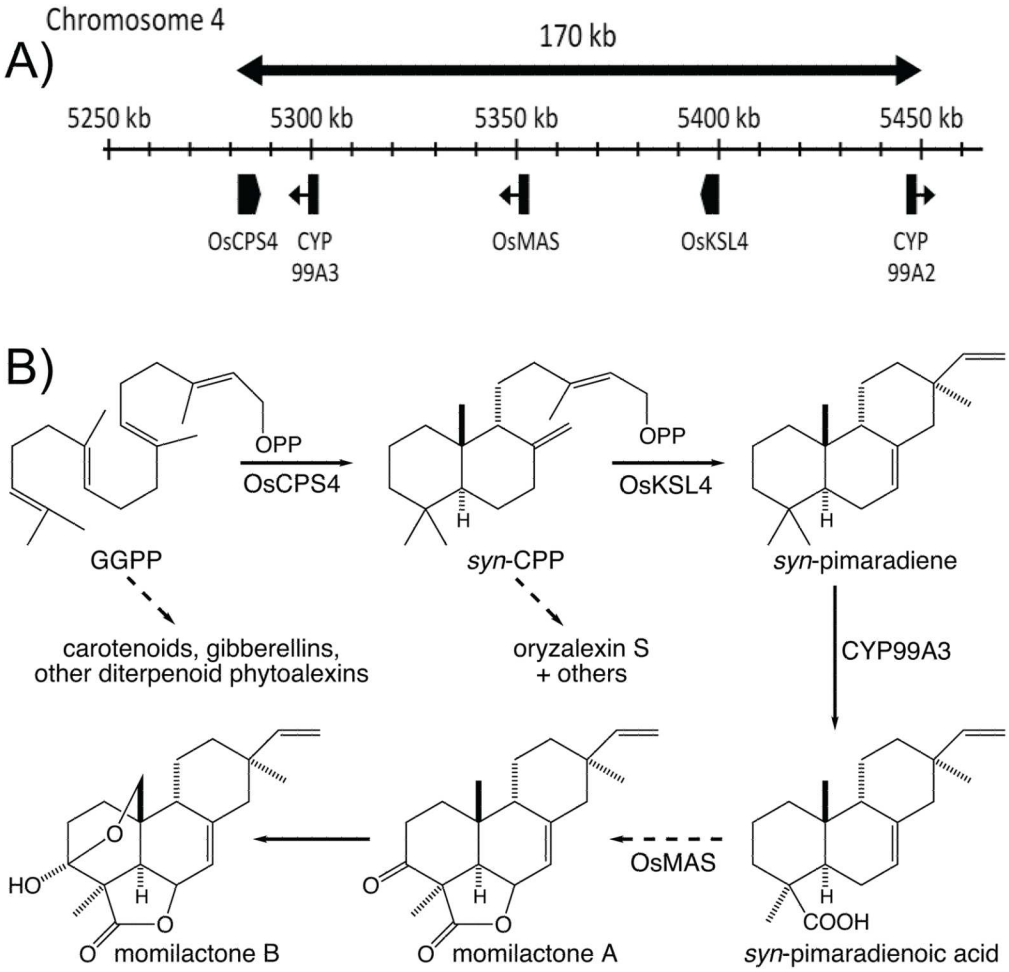

Fig. 1.
Rice momilactones. (a) Biosynthetic gene cluster; (b) biosynthetic pathway.

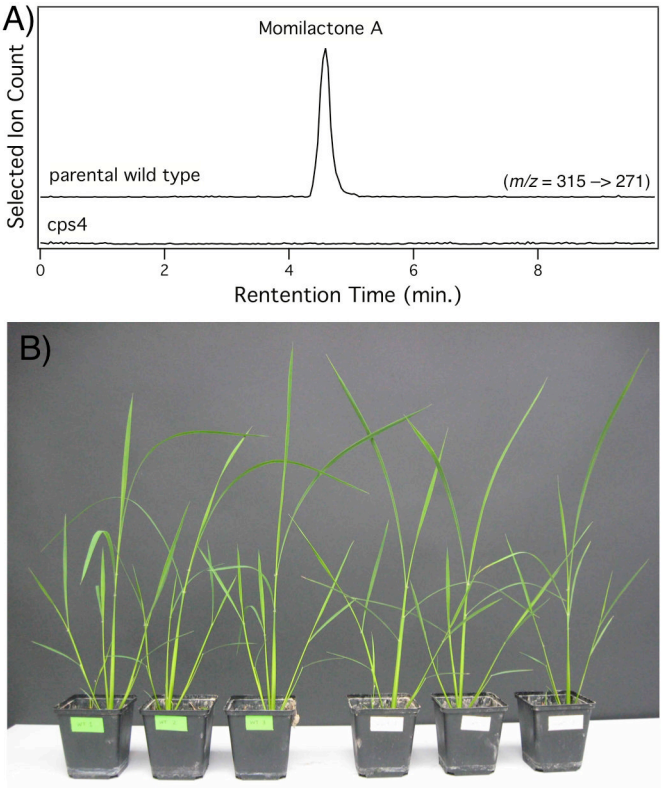

Fig. 2.

Effect of OsCPS4 knock-out (cps4) relative to parental wild-type (WT). (a) Loss of momilactone A in root exudates. LC-MS selected ion ($m/z = 315 \rightarrow 271$) chromatogram of root exudate extracts of eight 2-wk-old *Oryza sativa* seedlings for each genotype (as indicated). Note that these are shown on the same scale (see also Supporting Information Fig. S3). (b) Lack of effect on growth and development. Depicted are 4-wk-old seedlings: WT, green labels; cps4, white labels.

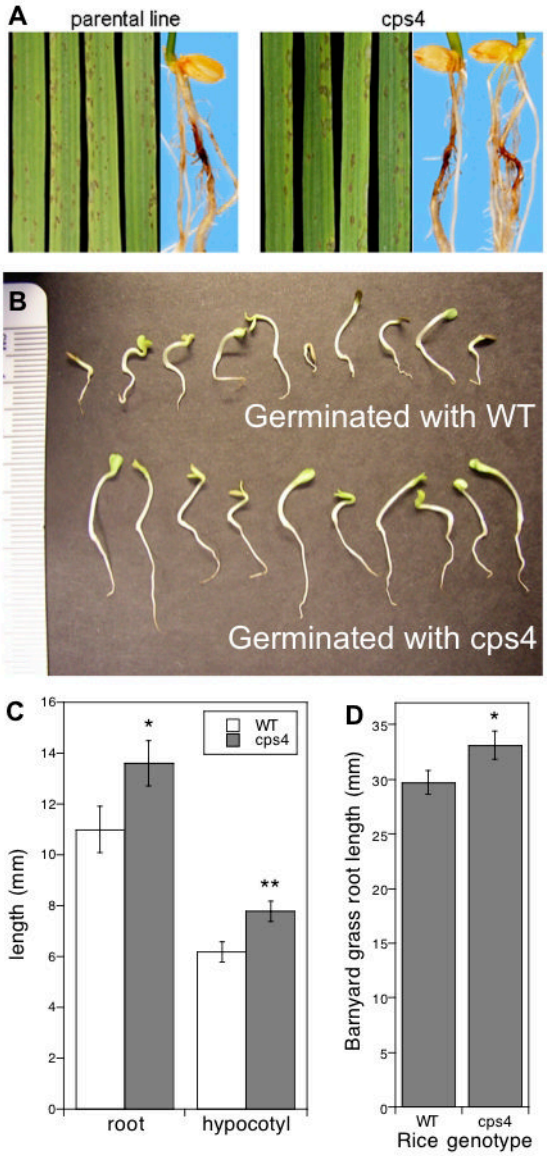

Fig. 3.

Effect of OsCPS4 knock-out mutant (cps4) on blast disease resistance and allelopathy. (a) Parental wild-type (WT) and cps4 mutant (as indicated) rice (*Oryza sativa*) plant leaves and roots infected with *Magnaporthe oryzae*. (b) Lettuce (*Lactuca sativa*) seedlings germinated in the presence of WT or cps4 rice seedlings (as indicated). (c) Comparison of allelopathic effect of WT (open bars) versus cps4 (closed bars) rice seedlings on growth of lettuce, both roots and hypocotyls (measured with 10 plates, totaling 60 lettuce seedlings, for each genotype). (d) Comparison of allelopathic effect of WT versus cps4 rice seedlings on root length of germinated barnyard grass (*Echinochloa crus-galli*) seedlings (measured with 10 plates, totaling 90 barnyard grass seedlings, for each genotype). For both (c and d) the histograms depict mean length (± SE). *P* values: *, < 0.05; **, < 0.005.

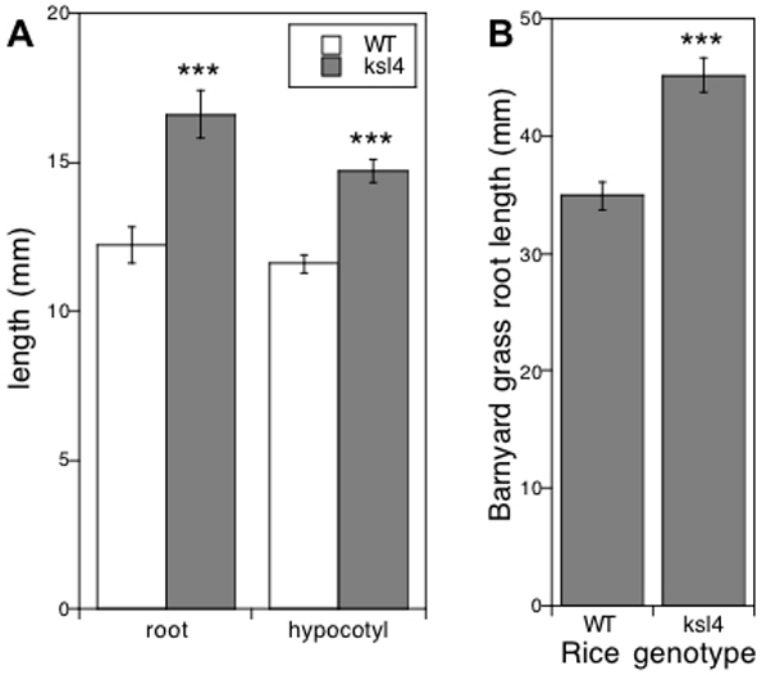

Fig. 4.

Effect of OsKSL4 knock-out mutant (ksl4) on allelopathy (histograms depict mean length, ± SE). All comparison between parental wild-type (WT) and ksl4 exhibit *P*-values < 0.001 (indicated by ***). (a) Comparison of allelopathic effect of WT (open bars) versus ksl4 (closed bars) rice (*Oryza sativa*) seedlings on growth of lettuce (*Lactuca sativa*), both roots and hypocotyls (measured with 10 plates, totaling 90 lettuce seedlings, for each genotype). (b) Comparison of allelopathic effect of WT versus ksl4 rice seedlings on root length of germinated barnyard grass (*Echinochloa crus-galli*) seedlings (measured with eight plates, totaling 80 barnyard grass seedlings, for each genotype).

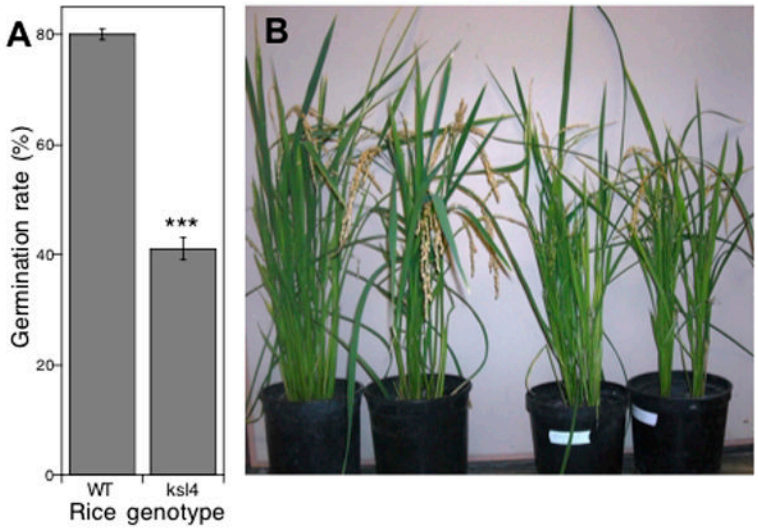

Fig. 5.

Effect of OsKSL4 knock-out mutant (ksl4) on germination and subsequent growth and development of rice (*Oryza sativa*). (a) Histogram showing reduced germination rate. This was measured in two separate trials, with 80 or 60 seeds, respectively, for each genotype (± SE). The associated *P*-value is < 0.001 (indicated by ***). (b) Lack of effect on subsequent growth and development. Depicted are mature 5-month-old plants: WT, left; ksl4, right (white labels).